PCT REQUEST

Original (for **SUBMISSION**)

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.163)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	**Applicant's or agent's file reference**	032796-247
I	**Title of Invention**	METHOD OF SYNTHESIZING AND PURIFYING DKK PROTEINS AND DKK PROTEINS OBTAINED THEREBY
II	**Applicant**	
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II-9	Facsimile No.	781.398.2530

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III-5-7	State of residence	US
III-6	**Applicant and/or inventor**	
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IV-1	**Agent or common representative; or address for correspondence**	
	The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	REA, Teresa, Stanek
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IV-1-4	Facsimile No.	703.836.2021
IV-1-6	Agent's registration No.	30,427
IV-2	**Additional agent(s)**	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	MEYER,Mercedes, K.(44,939)
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	

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VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	23 March 2004 (23.03.2004)	
VI-1-2	Number	60/555,406	
VI-1-3	Country	US	
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	42	-
IX-3	Claims	5	-
IX-4	Abstract	1	✓
IX-5	Drawings	17	-
IX-7	TOTAL	70	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-13	Priority document(s)	Item(s) VI-1	-
IX-17	PCT-SAFE physical media	-	✓
IX-18	other	Transmittal Letter, Postcard, Check	
IX-19	**Figure of the drawings which should accompany the abstract**	1	
IX-20	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**		
X-1-1	Name (LAST, First)	REA, Teresa, Stanek	
X-1-2	Name of signatory		
X-1-3	Capacity		

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10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

PCT (ANNEX - FEE CALCULATION SHEET)

Original (for **SUBMISSION**)

(This sheet is not part of and does not count as a sheet of the international application)

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	Date stamp of the receiving Office	

0-4	**Form PCT/RO/101 (Annex)** **PCT Fee Calculation Sheet**				
0-4-1	Prepared Using		PCT-SAFE [EASY mode] Version 3.50 (Build 0002.163)		
0-9	**Applicant's or agent's file reference**		032796-247		
2	**Applicant**		OSCIENT PHARMACEUTICALS CORPORATION		
12	**Calculation of prescribed fees**		fee amount/muliplier	Total amounts (USD)	
12-1	Transmittal fee	T	⇨	300	
12-2-1	Search fee	S	⇨	1920	
12-2-2	International search to be carried out by		EP		
12-3	International filing fee (first 30 sheets)	i1	1134		
12-4	Remaining sheets		40		
12-5	Additional amount	(X)	12		
12-6	Total additional amount	i2	480		
12-7	i1 + i2 =	I	1614		
12-12	EASY Filing reduction	R	-81		
12-13	Total International filing fee (i-R)	I	⇨	1533	
12-14	Fee for priority document Number of priority documents requested		0		
12-15	Fee per document	(X)	20		
12-16	Total priority document fee:	P	⇨		
12-17	**TOTAL FEES PAYABLE (T+S+I+P)**		⇨	3753	
12-19	**Mode of payment**		cheque		
12-20	**Deposit account instructions** The receiving Office		United States Patent and Trademark Office (USPTO) (RO/US)		
12-20-2	Authorization to charge any deficiency or credit any overpayment in the total fees indicated above		✓		
12-21	Deposit account No.		02-4800		
12-22	Date		23 March 2005 (23.03.2005)		
12-23	Name and signature		REA, Teresa, Stanek		

FIG. 12. Elution profiles of Dkk1 in PBS containing 0.05% N-BOG and 0.005% Tween-20. (▲) 0.5 mg or (●) 1 mg of Dkk1 purified in the presence of 0.7% N-BOG 0.1% Tween were subjected to preparative size exclusion chromatography (Superose-12, 10 x 300 mm column). Fractions (0.4 mL) were collected.

FIG. 13. SEC-MALLS Analysis of Dkk1. Dkk1 purified in the presence of 0.7% N-BOG (*panel A*) 0.1% Tween (*panel B*) was injected into a Bio-Sep 2000 (Phenomenex) column using PBS as the mobile phase and a flow rate of 0.5 ml/min. Protein was detected using a Wyatt optilab DSP refractometer, a Wyatt Dawn EOS, and an Agilent 1100 series photo-diode array. Panels C and D represent the molar mass vs. volume and cumulative molar mass profiles, respectively, as determined from the Astra 4.90.07 software.

FIG. 14. Analytical ultracentrifugation analysis of Dkk1. Dkk1 was subjected to sedimentation velocity analysis of HEK293 EBNA derived Dkk1 centrifuged at 35,000 RPM at 20°C. Top, middle and lower panels correspond to Sed-Vel analyses of the Dkk1 protein in PBS buffer containing 0.001% Tween, PBS alone and PBS containing 0.03% BOG. Sedimentation equilibrium analysis of Dkk1 (panels C and D) was performed using a Beckman XL-I analytical ultracentrifuge using 6-sector cells. Panels C and D represent the sedimentation profiles of Dkk1 centrifuged at 35 krpm. Panel C and D contains 1.66 uM Dkk1 containing 0.002% Tween-20 and 0.012% N-BOG, respectively.

FIG. 15. Nucleic acid (A; SEQ ID NO:4) and amino acid (B; SEQ ID NO:5) sequences of human Dkk1 as used to obtain purified Dkk1 from the HEK293 EBNA cells *infra*. Open reading frames (ORF) are in bold in FIG 15A. The secretion signal peptide of Dkk1 is in bold and underlined in FIG. 15B. The c-myc tag is in italics and underlined and His_6-tag is double underlined in FIG. 15A.

Detailed Description of the Invention

1. Definitions and Acronyms

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a

EXAMPLES

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention, and would be readily known to the skilled artisan.

Example 1

Synthesis of Dkk1

HEK293T cells (ATCC Cat. No. CRL11268) were plated at 90-95% confluency in DMEM media supplemented with 10% fetal calf serum. Specifically, cells were trypsinized and plated the day before transfections at 9.6 x10^6 cells per T75 flask. Human Dkk1 (GenBank Accession No. AF177394) was cloned into the pcDNA3.1/*myc*-His vector (Invitrogen) containing c-*myc* and His6 tags at the carboxy terminus as follows. A clone containing the full length Dkk1 open reading frame in the pC52 vector was subjected to PCR using PFU Turbo Polymerase (Stratagene, Cat. No. 600250). 5'-TTTTTTGGATCCGCCACCATGATGGCTCT GGGCGCAG-3' (SEQ ID NO:1) was used as forward primer and 5'-TTTTTTTCTAGAGTGTCTC TGACAAGTGTG-3' (SEQ ID NO:2) was used as reverse primer. The 0.8 kb PCR product was purified from an agarose gel using the Qiaex II gel extraction kit (Qiagen Cat. No. 20021) as specified by the manufacturer. The purified PCR product was digested with *Bam*HI and *Xba*I and ligated to pcDNA3.1/*myc*-His (Invitrogen Cat. No. V800-20) which was linearized with the same enzymes. The ligation mix was transformed into ElectroMAX DH10B cells (Invitrogen Cat. No. 18290-015) according to the manufacturer's instructions; clones containing the plasmid were isolated and amplified in LB Broth containing 100 µg/ml ampicillin. The clones were sequenced to confirm the correct sequence and that the Dkk1 and *myc*-His sequences were in the same open reading frame.

HEK293T cells were transfected with the pcDNA3.1 vector containing the nucleic acid expressing Dkk1 as follows. Twelve µg of pDNA3.1-Dkk1*myc*his DNA was diluted into 900 µL of OPTI-MEM media (Invitrogen). Then, for each T75 flask, 45 µL LIPOFECTAMINE 2000® (Invitrogen, Cat. No. 11668) was diluted into 900 µL of OPTI-MEM and then incubated for about 5 minutes at room temperature. Once the diluted LIPOFECTAMINE 2000® is prepared, it must be combined with the diluted DNA within 30 minutes of its preparation. However, this LIPOFECTAMINE 2000® procedure can be done in bulk for multiple flasks. After combining the diluted DNA with the diluted LIPOFECTAMINE 2000®, the mixture

per well of a 96-well plate. After 24 hours of incubation at 37°C (cells were 80-90% confluent at that point), the media was replaced with 100 µL fresh serum free OPTIM media (Gibco/BRL). Both cell types were transfected with 16xTCF (TK)-firefly luciferase (0.3 µg/well), TK-renilla luciferase (0.06 µg/well) using LIPOFECTAMINE 2000® transfection Reagent (Promega, Madison, WI) pursuant to manufacturer's instructions. The DNA mix and reagent were then incubated for 30 minutes and 50 µL/well of the DNA-reagent mix was added to 100 µL of OPTIM and incubated for 4 hours at 37°C. The transfection medium was then replaced and 140 µL of fresh DMEM or RPMI media was added to the HEK293A and U2OS cells respectively. After 20-24 hours of incubation at 37°C in a CO_2 incubator, the media was removed. The transfected cell monolayer was lysed using 150 µL of 1X lysis buffer of Dual Luci reagent (Promega Corp., Madison, WI). After 10 min., 20 µL of the lysate was transferred into a 96-well white plate (Packard/Costar). Cell lysates were mixed with 100 µL/well of LARII buffer (Dual Luci Reagent) and the Relative Luciferase Units (RLUs) were measured. This was followed by the addition of 100 µL/well of "stop & glo" reagent (Dual Luci reagent) and the internal control renilla luciferase was measured. The ratio of TCF-firefly luciferase to renilla was calculated and the activity is indicated in Fig. 10 as +/-.

Assessment of the mutants' ability to interact with LBD1 and LBD4 of LRP5 was assessed using a yeast two hybrid assay. The deletions showed that although binding with the LBD domains may remain as tested in the yeast two hybrid assay, the Dkk1 function was lost if the C-terminal 21 amino acids (*i.e.*, N-RIQKDHHQASNSSRLHTCQRH-C) (SEQ ID NO:3) was missing when tested by the TCF assay.

Example 5

Inhibition of Wnt3a Activity by Dkk1

Another characteristic of the Dkk1 purified from HEK293T cells is its ability to inhibit Wnt3A mediated signaling. The purified HEK293T-derived Dkk1 was compared with Dkk1 from other sources to test the effect of purification method on Dkk1 activity.

The Dkk1 proteins used were as follows: 1) Recombinant human Dkk1 from R&D systems (Cat. No. 1096-DK/CF); 2) inclusion body Dkk1 solubilized in urea and renatured; 3) inclusion body Dkk1 solubilized in GuCl Dkk1 and renatured; 4) Dkk1 from conditioned media prepared from HEK293T cells transiently transfected with pcDNA*myc*HisDkk1 – the wild-type gene; 5) Dkk1 from conditioned media